HIGHLY PERMISSIVE CELL LINES FOR HEPATITIS C VIRUS REPLICATION

Statement Regarding Federally Sponsored Research or Development.

This invention was made with U.S. Government support under grant numbers CA57973 and AI40034. The U.S. Government may have certain rights in this invention.

Background

1. Field of the Invention

This invention relates generally to cells that are permissive for hepatitis C virus (HCV) replication, and methods and materials for making and using them.

2. Related Art

Adaptive mutations in the HCV non-structural proteins that increase RNA replication, and the frequency of Huh-7 cells supporting detectable levels of replication, have been identified previously. (Blight, K.J. et al, Science 290:1972-1974, 2000; Guo, J.T. et al., J. Virol. 75:8516-8523, 2001; Kreiger, N. et al., J. Virol. 75:4614-4624, 2001; Lohmann, V. et al., J. Virol. 75:1437-1449, 2001). In particular, replacement of the Serine residue with Isoleucine at position 2204 in NS5A permits HCV replication in ~10% of transfected Huh-7 cells (a 20,000-fold improvement over non-mutated HCV) and increases replication to a level sufficient for the detection of HCV RNA early after transfection. (Blight et al., 2000). The low frequency of Huh-7 cells supporting HCV replication suggests that the cellular environment may be a major determinant of HCV replication efficiency.

Brief Description of the Drawings

FIG. 1 shows schematic representations of various HCV RNAs.

FIG. 2 shows selection of cell lines highly permissive for HCV replication.

FIG. 3 shows detection of HCV proteins and RNA in Huh-7.5 and Huh-7 cells transiently transfected with HCV RNA.

FIG. 4 shows HCV RNA accumulation after transfection of Huh-7.5 cells with full-length HCV RNA.

FIG. 5 shows effects of alternative substitutions at position 2204 on HCV RNA replication.

FIG. 6 shows effects of combining NS5A adaptive mutations on HCV Rna replication.

FIG. 7 shows effects of combining NS3 and NS5A mutations on HCV replication.

FIG. 8 shows effect of S2194A and S2194D mutations on HCV RNA replication.

Description

As used herein the term "permissive" for HCV replication, in reference to a particular cell or cell line, means that the particular cell or cell line supports HCV replication at a frequency that is greater than that of the cell or cell line from which it was derived. For example, in certain embodiments described herein, Huh-7 sublines support a higher frequency of HCV replication than the Huh-7 cell line from which they were derived. Thus, the sublines are said to be permissive for HCV replication. In some embodiments, the cell or cell line supports replication of HCV at a frequency of between about 10% to about 30%. In other embodiments, the cell or cell line supports replication of HCV at a frequency of between about 10% and about 75%.

The term "cured" refers to cells substantially free of self-replicating HCV RNA. Example 1 provides a description of one means for curing cells, within the meaning of that term as used herein.

The term "transfection" refers to the infection of a cell with a polynucleotide. The polynucleotide may be either DNA or RNA. Methods of transfecting a cell are known in the art, any of which may be used.

Frequency is ascertained by determining the percent of cells having replicating HCV RNA. One easy way to measure frequency is to determine the percentage of cells that exhibit a characteristic conferred by the HCV RNA, and any method known in the art for accomplishing this is suitable. The examples herein describe such a method utilizing HCV RNA comprising a *neo* gene and G418 selection.

Unless otherwise noted, conventional techniques of cell culture, molecular biology, microbiology, recombinant DNA and immunology are employed, all of which are within the skill of the art and are described in the literature.

In order to obtain cell lines permissive for HCV replication, clonal and population Huh-7 cell lines supporting adapted and non-adapted subgenomic or full-length RNA replication were cured of HCV RNA by treatment with interferon (IFN). Since HCV replication can be readily blocked by IFN, prolonged treatment with IFN cures cells of HCV RNA. A higher percentage of cured cells were able to support HCV replication and facilitated the detection of both subgenomic and full-length replication by multiple assays. Thus, one embodiment described herein comprises a method of making cells and cell lines that are permissive for HCV replication. Such a method comprises curing HCV infected cells. The cured cells may then be assayed to determine the frequency at which cells support HCV replication.

The cells may be any vertebrate cells that are capable of supporting adapted or non-adapted HCV RNA replication. The methods described herein for making cells and cell lines are believed to be applicable to any cell type that is capable of supporting adapted or non-adapted HCV RNA replication. Such cells may include, for example, hepatocytes, T-cells, B-cells, or foreskin fibroblasts, and may be mammalian or more specifically human cells. A particularly useful cell type is hepatocyte cells. For example, Huh-7 cells have been shown to support HCV RNA replication.

It is demonstrated herein that Huh-7 sublines that have been cured are permissive for HCV RNA replication. For the replicon containing the highly adaptive NS5A S2204I mutation, at least 30% of the Huh-7.5 cells can be transduced to G418 resistance. A comparable fraction was positive for the NS3 antigen by FACS. Similarly, for the SG replicons lacking *neo* (Fig. 1), at least 50% initiation efficiency was achieved. Data from more sensitive FACS analysis (data not shown) indicates that >75% of the cells that survive the transfection procedure harbor replicating HCV RNAs. These permissive cells were obtained by curing replicon-containing cell clones with IFN.

Other embodiments comprise methods of making cells and cell lines that are permissive for HCV replication. Such methods comprise curing infected cells, and

subsequently assaying sublines to determine the frequency with which a particular subline supports HCV replication. Sublines that are particularly permissive may then be identified. The infected cells may be cured by any means. For example, treatment with interferon-α is an effective means of curing cells of HCV infection, although any effective means of curing may be utilized within the scope of this invention.

The most highly permissive sublines (Huh-7.5 and Huh-7.8) were obtained from G418-selected clones that harbored replicons without adaptive changes in the NS3-5B region (at the population sequence level). These cells may represent a subpopulation of the original Huh-7 parental line that are permissive for replication of unmodified replicons as well as more permissive for replicons with adaptive mutations. Curing of other replicon-containing cell lines did not always yield a cell population that was more permissive for the replicons tested. For instance, curing the Huh-7 population containing the SG-Neo (S2204I) replicon, yielded a cell substrate that was unchanged in its ability to support SG-Neo (S2204I), but less efficient (23-fold) for initiation of SG-Neo (5AΔ47) (Fig. 2).

The ability to study HCV replication directly after transfection, without the need for G418 selection, allowed for examination of replication of subgenomic replicons lacking *neo* as well as full-length HCV genome RNAs. Initial attempts to create a monocistronic replicon by fusing cellular ubiquitin in-frame between the first 12 amino acids of core and NS3 {SG-5'Ub-NS3 (S2204I); Fig. 1} were unsuccessful. A bicistronic derivative with ubiquitin fused to NS3-5B was viable, suggesting that the failure of SG-5'Ub-NS3 (S2204I) to replicate was not due to a defect in processing at the ubiquitin/NS3 junction (data not shown). Rather, the fusion of the ubiquitin-coding sequence near the HCV 5' NTR may have interfered with translation due to the formation of deleterious RNA secondary structures or RNA replication, by disrupting RNA elements that lie in the HCV 5' NTR or its complement. Fusion of the HCV 5' NTR to the EMCV IRES yielded a subgenomic replicon that replicated better than SG-Neo (S2204I) (Fig. 3). Why deletion of the first cistron (sequences encoding C-Neo fusion) from the bicistronic SG-Neo (S2204I) stimulated replication is unknown, but may result from enhanced translation of the replicase due to abrogated binding of the 40S ribosomal subunit to the usual HCV translation initiation

site and diminished competition between the EMCV and HCV IRES elements (J. Marcotrigiano and C. M. Rice, unpublished results).

A similar picture was observed for replication of the full-length constructs containing the NS5A S2204I adaptive change (Fig. 4). In Huh-7.5 cells, the bicistronic construct containing the C-Neo cistron {FL-Neo (S2204I); Fig. 1} initiated replication less efficiently than the RNA with the HCV 5' NTR fused to the EMCV IRES {FL-5'HE (S2204I); Fig. 1}. The FL construct with the unmodified HCV genome (except for the S2204I substitution in NS5A) was better at initiating replication than RNA where translation was mediated by the EMCV IRES (Fig. 4), demonstrating that EMCV IRES-driven translation is not required for HCV replication in Huh-7.5 cells, thus allowing the study of unmodified HCV genomic RNAs. This latter point could certainly impact the ability of HCV RNAs to be packaged into infectious particles. However, in our hands (K. J. Blight and C. M. Rice, unpublished results) and in a recent report (Pietschmann, T. et al., J. Virol. 76:4008-4021, 2002) selective packaging of these unmodified FL RNAs was not observed in Huh-7 cells. Full-length HCV RNAs were less efficient at establishing replication than the corresponding adapted subgenomic replicons, suggesting that addition of the structural-NS2 coding region inhibits HCV replication initiation. Whether this is due to the encoded proteins or RNA elements that lie in this region (or both) (3-1) is currently unclear. We have observed that the levels of HCV replication (as measured by HCV protein and RNA levels) of S2204I-containing FL and FL-Neo RNAs, but not subgenomic replicons, is dependent upon the cell cycle which might compromise the ability of the full-length RNAs to initiate replication in an unsynchronized transfected cell population (Balfe et al., submitted).

In an attempt to further enhance HCV replication in cell culture, the effect of other amino acid substitutions at position 2204 in NS5A was examined. Efficient subgenomic RNA replication was observed for Ile and Val and to a much lesser extent, Ala at position 2204 (Fig. 5). Val or Ile are small, β-branched, non-polar residues, whereas Ala has similar properties, but is not β-branched. In contrast, polar residues such as Tyr, Glu, Thr, Ser or Asp at position 2204 severely impaired HCV replication (Fig. 5), suggesting that replication favors non-polar residues at this locus. It is interesting to note that Ser is found naturally at position 2204 for this genotype 1b

isolate (Lohmann, V. et al., Science 285:110-113, 1999) and is conserved between other HCV genotypes (Tanji, Y. et al., J. Virol. 69:3980-3986, 1995), suggesting that this residue may be important for HCV replication and/or pathogenesis in vivo.

Combining NS5A adaptive mutations resulted in replicons that were either impaired (A2199T+S2204I) or unable to replicate (S2197P+A2199T+S2204I) in Huh-7.5 cells (Fig. 6). Incompatability of adaptive mutations elsewhere in the HCV NS coding region has been previously described (Lohmann, V. et al., J. Virol. 75:1437-1449). For example, combining an adaptive mutation in NS5B (R2884G) with either NS4B (P1936S) or NS5A (E2163G) drastically reduced the efficiency of G418-resistant colony formation. On the other hand, combining certain NS3 and NS5A adaptive mutations can increase replication efficiency (Krieger, N. et al., J. Virol. 75:4614-4624, 2001). However, despite the observation that mutations E1202G and T1280I in NS3 act synergistically with S2197P in NS5A to increase the replication efficiency (Krieger et al., 2001, and K. J. Blight and C. M. Rice, unpublished results), engineering these NS3 changes into SG-5'HE (S2204I) did not enhance replication in our system (Fig. 7). These results again underscore the empirical nature of optimizing adaptive mutations with different Huh-7 cellular environments.

The phosphorylation of NS5A is conserved among divergent HCV genotypes (Reed, K.E. et al, J. Virol. 71:7187-7197, 1997) suggesting that it plays an important role in the virus life cycle. We previously showed that NS5A hyperphosphorylation is not essential for HCV replication (Blight, K.J. et al., Science 290:1972-1974, 2000). Following the recent identification of S2194 as a major phosphate acceptor site for subtype 1b (Katze, M.G. et al, Virology 278:501-513), Ala or Asp was substituted at this position and the effect on HCV replication was examined in the context of the S2204I adaptive mutation. Given the incompatibilities observed when combining NS5A mutations, the absolute replication efficiencies of the different mutants could not be evaluated, however replicating RNAs were recovered that harbored these substitutions at the 2194 locus. These results show that phosphorylation at S2194 is not an absolute requirement for replication of this subtype 1b isolate.

Various embodiments of the invention are described in the following examples. These examples are to be considered exemplary only, and are not intended to be limiting.

Examples

EXAMPLE 1: Cell culture and interferon treatment.

Huh-7 cell monolayers were propagated in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.1 mM non-essential amino acids (DMEM-10% FBS). For cells supporting subgenomic replicons, 750 μg/ml G418 (Geneticin; Gibco-BRL) was added to the culture medium. Replicon-containing Huh-7 cells were cured of HCV RNA by initially passaging cells twice in the absence of G418. On the third passage cells were cultured with 100 IU/ml of human leukocyte-derived IFN (Sigma-Aldrich). After 3-4 days, confluent monolayers were trypsinized, plated and cultured for 24 h before the addition of IFN. Cells were passaged a total of four times in the presence of IFN and prior to the fourth passage cells were grown for 3 days without IFN. Cured cell lines were expanded and cryopreserved at early passage levels. Further experiments were conducted using cells that been passaged less that 20-30 times from these cryopreserved seed lots.

EXAMPLE 2: Plasmid construction.

Standard recombinant DNA technology was used to construct and purify all plasmids. Primed DNA synthesis was performed with Klen*Taq*LA DNA polymerase (kindly provided by Wayne Barnes, Washington University, St. Louis), and regions amplified by PCR were confirmed by automated nucleotide sequencing. Plasmid DNAs for in vitro transcription were prepared from large-scale bacterial cultures and purified by centrifugation in CsCl gradients.

All nucleotides (nt) and amino acid numbers refer to the location within the genotype 1b Con1 full-length HCV genome (Genbank Accession no. AJ238799) commencing with the core-coding region. This sequence was assembled from chemically synthesized DNA oligonucleotides in a step-wise PCR assay essentially as described previously (5). Briefly, 10-12 gel-purified oligonucleotides (60-80 nt in

length) with unique complementary overlaps of 16 nt were used to synthesize cDNAs spanning 600-750 bases. The final PCR products were purified, digested with appropriate restriction enzymes, and ligated into the similarly cleaved pGEM3Zf(+) plasmid vector (Promega). Multiple recombinant clones were sequenced, correct clones identified and overlapping cDNA fragments assembled into the contiguous genomic sequence: 5'NTR-C-E1-E2-p7-NS2-3-4A-4B-5A-5B-3'NTR (pHCVBMFL). The selectable replicon, pHCVrep1bBartMan/AvaII {SG-Neo (wt); Fig. 1} and the derivatives, pHCVrep1b/BBVII {SG-Neo (S2204I)} and pHCVrep1b/BBI {SG-Neo (5AΔ47)}, containing the NS5A adaptive mutations, S2204I and an in-frame deletion of 47 amino acids (Δ47aa) between nt 6960 and 7102, respectively, have been described (5) (Fig. 1). The plasmid pHCVBMFL/S2204I {FL (S2204I); Fig. 1} contains the full-length genome with the NS5A adaptive change S2204I. For the genomic and subgenomic constructs, NS5B polymerase defective derivatives were generated carrying a triple amino acid substitution, changing the Gly-Asp-Asp (GDD) motif in the active site to Ala-Ala-Gly (AAG) (5), and throughout this report are referred to as pol$^-$.

The plasmid pC-Ubi-NS3/HCVrepBBVII {SG-5'Ub-NS3 (S2204I); Fig. 1} containing ubiquitin instead of the *neo* gene and EMCV IRES was constructed as follows. An *Asc*I-*Sac*I digested PCR fragment amplified from pHCVrep12/Neo (Blight et al., unpublished results) with primers 1289 and 1290 (Table 1) and the *Sac*I-*Bsr*GI portion of a second PCR product generated using the primer pair 1291/1292 (Table 1) with pHCVrep1b/BBVII were ligated between the *Xba*I and *Bsr*GI sites of HCVrep1b/BBVII together with the *Xba*I-*Asc*I fragment from HCVrep1b/BBVII. To delete the *neo* gene from pHCVrep1b/BBVII, synthetic overlapping oligonucleotides 1287 and 1288 (Table 1) were hybridized and extended to create the junction between the 5' NTR and the EMCV IRES. This product was digested with *Apa*LI and *Acl*I, and inserted, together with *Xba*I-*Apa*LI and *Acl*I-*Eco*RI fragments from pHCVrep1b/BBVII, into *Xba*I-*Eco*RI digested pHCVrep1b/BBVII. This construct was named p5'NTR-EMCV/HCVrepBBVII {SG-5'HE (S2204I); Fig. 1}. To replace S2204I with NS5AΔ47, the *Eco*RI-*Xho*I fragment from pHCVrep1b/BBI was ligated into similarly cleaved p5'NTR-EMCV/HCVrepBBVII, generating p5'NTR-EMCV/HCVrepBBI {SG-5'HE (5AΔ47); Fig. 1}.

The plasmid p5'NTR-EMCV/HCVFLBM(S2204I) {FL-5'HE (S2204I); Fig. 1} was created by ligating the *Xba*I-*Hind*III fragment from p5'NTR-EMCV/HCVrepBBVII, the *Hind*III-*Aat*II fragment of a PCR product amplified from p5'NTR-EMCV/HCVrepBBVII using primers 1293 and 1294 and the *Aat*II-*Not*I fragment from pHCVBMFL/S2204I into pHCVBMFL/S2204I previously digested with *Xba*I and *Not*I. The selectable bicistronic full-length HCV clone pHCVBMFL(S2204I)/Neo {FL-Neo (S2204I); Fig.1} was assembled by ligating the *Xba*I-*Hind*III fragment from pHCVrep1b/BBVII and the *Hind*III-*Eco*RI fragment from p5'NTR-EMCV/HCVBMFL(S2204I) between the *Xba*I and *Eco*RI sites of pHCVrep1b/BBVII.

To obtain plasmids with mutations at position 2204, and to introduce single A2199T or double S2197P/A2199T mutations into p5'NTR-EMCV/HCVrepBBVII, PCRs were first performed using p5'NTR-EMCV/HCVrepBBVII as a template with the reverse primer 1030 and one of the following mutant forward primers: 1319 (S2204V), 1320 (S2204A), 1322 (S2204Y), 1324 (S2204E), 1325 (S2204T), 1184 (S2204D), 1326 (A2199T+S2204I) and 1327 (S2197P+A2199T+S2204I) (Table 1). PCR-amplified products were digested with *Blp*I and *Xho*I and cloned into these sites in p5'NTR-EMCV/HCVrepBBVII. S2204 was engineered by insertion of the *Eco*RI-*Xho*I fragment from pHCVrep1bBartMan/AvaII into similarly cleaved p5'NTR-EMCV/HCVrepBBVII.

To engineer the mutation, Q1112R, into p5'NTR-EMCV/HCVrepBBVII in order to create p5'NTR-EMCV/HCVrepCloneA (Q1112R+S2204I), nt 3640-3991 of NS3 were PCR amplified from p5'NTR-EMCV/HCVrepBBVII using mutant primer 1358 and oligonucleotide 885 (Table 1). The resulting product was digested with *Bsr*GI and *Eag*I and combined in a ligation reaction mixture with the *Eag*I-*Eco*RI and *Bsr*GI-*Eco*RI fragments from p5'NTR-EMCV/HCVrepBBVII. The double mutation (E1202G+T1280I) in NS3 was created via a multistep cloning procedure. First, a PCR fragment amplified from p5'NTR-EMCV/HCVrepBBVII with forward primer 1359 and reverse primer 1356 (Table 1) was digested with *Apa*LI and *Xba*I and cloned into *Eco*RI-*Xba*I digested pGEM3Zf(+) together with the *Eco*RI-*Apa*LI fragment from pGEM3Zf(+)/HCV1bnt1796-2524, containing nt 3420-4124 in NS3 (K. J. Blight and C. M. Rice, unpublished results), generating the intermediate

plasmid pGEM3Zf(+)/HCV1bnt1796-2524NS3*. Second, in a four part cloning strategy, the *Bsr*GI-*Bsa*AI fragment, excised from pGEM3Zf(+)/HCV1bnt1796-2524NS3*, was inserted, together with fragments *Bsa*AI-*Bss*HII and *Bss*HII-*Eco*RI from p5'NTR-EMCV/HCVrepBBVII, into p5'NTR-EMCV/HCVrepBBVII cleaved with *Bsr*GI and *Eco*RI. The resultant plasmid was named p5'NTR-EMCV/HCVrepBBVII+NS3* (E1202G+T1280I+S2204I).

The mutations S2194A and S2194D were introduced by using primer pairs 5'Ala/1030 and 5'Asp/1030 (Table 1), respectively to PCR amplify nt 6897-7186 in NS5A from pHCVrep1b/BBVII. These mutations were incorporated into pHCVrep1b/BBVII by replacing the *Blp*I-*Xho*I portion with the corresponding *Blp*I-*Xho*I digested PCR product.

EXAMPLE 3: RNA transcription.

Plasmid DNAs containing full-length and subgenomic HCV sequences were linearized with *Sca*I and a poliovirus subgenomic replicon digested with *Bam*HI. The linearized DNAs were phenol:chloroform (1:1) extracted, and precipitated with ethanol. Pelleted DNAs were washed in 80% ethanol and resuspended in 10 mM Tris-HCl (pH 8.0)/1 mM EDTA (pH 8.0). RNA transcripts were synthesized at 37°C for 90 min in a 100 µl reaction mixture containing 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 12 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol (DTT), 3 mM of each nucleoside triphosphate, 0.025 U of inorganic pyrophosphatase (Roche Applied Science), 100 U of RNasin (Promega), 100 U of T7 RNA polymerase (Epicentre Technologies), and 2 µg of linearized DNA. RNA was extracted with phenol-chloroform (1:1), ethanol precipitated, and the pellet washed in 80% ethanol before resuspension in ddH_20. DNA template was removed by three serial DNase digestions for 20 min at 37°C in 33 mM Tris-HCl (pH 7.8), 66 mM KCl, 10 mM $MgCl_2$ and 5 mM DTT containing 10 U of DNase I (Roche Applied Science). DNase-digested RNAs were extracted with phenol:chloroform (1:1), ethanol precipitated and the RNA pellet resuspended in ddH_20 after washing in 80% ethanol. The RNA concentration was determined by measurement of the optical density at 260 nm and the integrity and concentration confirmed by 1% agarose gel electrophoresis and ethidium bromide staining.

EXAMPLE 4: Transfection of cultured cells.

In vitro-transcribed RNA was transfected into Huh-7 and IFN-cured cells by electroporation. Briefly, subconfluent Huh-7 cells were detached by trypsin treatment, collected by centrifugation (500 x g, 5 min), washed three times in ice-cold RNase-free phosphate-buffered saline (PBS) and resuspended at 1.25 x 10^7 cells/ml in PBS. RNA transcripts (1 μg) were mixed with 0.4 ml of washed Huh-7 cells in a 2-mm gap cuvette (BTX) and immediately pulsed (0.92 kV, 99 μsec pulse-length, 5 pulses) using a BTX ElectroSquarePorator. Pulsed cells were left to recover for 10 min at room temperature (rt) and then diluted into 10 ml DMEM-10% FBS. Cells were plated in: (i) 35-mm diameter wells for quantifying HCV RNA and for metabolic labeling experiments; (ii) eight-well chamber slides (Becton Dickinson) for immunofluorescence studies or; (iii) 100-mm diameter dishes for fluorescent activated cell sorting (FACS) analysis and G418 selection. To determine the efficiency of G418-resistant colony formation, transfected cells were plated at multiple densities (between 1 x 10^3 and 2 x 10^5 cells), together with cells transfected with pol$^-$ RNA transcripts such that the total cell number was maintained at 2 x 10^5 cells per 100-mm diameter dish. Forty-eight hours after plating, medium was replaced with DMEM-10% FBS supplemented with 1 mg/ml G418. Three weeks later, G418 resistant foci were fixed with 7% formaldehyde and stained with 1% crystal violet in 50% ethanol to facilitate colony counting. The G418 transduction efficiency was calculated based on the number of G418-selected colonies relative to the number of Huh-7 cells plated after electroporation.

Transfection efficiency was monitored for each series of RNAs by electroporating in parallel a poliovirus subgenomic replicon expressing green fluorescent protein (GFP; A. A. Kolykhalov and C. M. Rice, unpublished results). Transfected cells were observed for poliovirus replicon-induced cytopathic effect and GFP expression visualized using a fluorescent inverted microscope at 12-16 h posttransfection. After 24 h, the surviving attached cells (presumably not transfected with the poliovirus replicon) were trypsinized, mixed with trypan blue and viable cells counted to determine the percentage of cells electroporated.

EXAMPLE 5: Viral RNA analysis.

Total cellular RNA was isolated using TRIZOL reagent (Gibco-BRL) according to the manufacturer's protocol. One-tenth of each RNA sample was used to quantify HCV-specific RNA levels using an ABI PRISM 7700 Sequence Detector (Applied Biosystems). Real time reverse transcription (RT)-PCR amplifications were performed using the TaqMan EZ RT-PCR core reagents (Applied Biosystems) and primers specific for the HCV 5' NTR: 5'-CCTCTAGAGCCATAGTGGTCT-3' (sense, 50 μM), 5'CCAAATCTCCAGGCATTGAGC-3' (antisense, 50 μM) and FAM-CACCGGAATTGCCAGGACGACCGG (probe, 10 μM; Applied Biosystems). RT reactions were incubated for 30 min at 60°C, followed by inactivation of the reverse transcriptase coupled with activation of *Taq* polymerase for 7 min at 95°C. Forty cycles of PCR were performed with cycling conditions of 15 sec at 95°C and 1 min at 60°C. Synthetic HCV RNA standards of known concentration were included with each set of reactions and used to calculate a standard curve. The real time PCR signals were analyzed using SDS v1.6.3 software (Applied Biosystems).

EXAMPLE 6: FACS anaysis.

Transfected cell monolayers were removed from 100-mm diameter culture dishes by versene/EDTA treatment and a single cell suspension prepared by passing cells through a 16-gauge needle and a 74 μm pore membrane. Cells were resuspended at 2 x 10^6 per ml, an equal volume of 4% paraformaldehyde added to the cell suspensions and incubated for 20 min at rt. Fixed cells were washed twice with PBS and the resultant cell pellet resuspended at 2 x 10^6 cells per ml in 0.1% saponin/PBS. After incubation for 20 min at rt, cells were stained (1 h at rt) with HCV-specific monoclonal antibodies (mAbs; core (C750), NS3 (1B6) and NS5B (12B7); all generously provided by Darius Moradpour, University of Freiburg, Freiburg, Germany) diluted to 10 μg/ml in 3% FBS/0.1% saponin/PBS. Cells were washed three times with 0.1% saponin/PBS and bound mAb detected by incubation for 1 h at rt with anti-mouse IgG conjugated to Alexa 488 (Molecular Probes) diluted 1:1000 in 3% FBS/0.1% saponin/PBS. Stained cells were washed three times with 0.1% saponin/PBS, resuspended in FACSflow buffer (BD Biosciences) and analyzed immediately using a FACS Calibur (BD Biosciences).

EXAMPLE 7: Indirect immunofluorescence.

Electroporated Huh-7.5 cells seeded in eight-well chamber slides were washed with PBS and fixed in 4% paraformaldehyde for 20 min at rt. Cells were washed twice with PBS, permeabilized by incubation with 0.1% saponin/PBS for 20 min at rt and blocked with 3% goat serum for 20 min at rt. The NS5B mAb (12B7) was diluted to 10 μg/ml in 0.1% saponin/3% goat serum/PBS and incubated for 1 h at rt, followed by three washes with 0.1% saponin/PBS. Bound mAb were detected by incubating for 1 h at rt with anti-mouse IgG conjugated to Alexa 488 diluted 1:1000 in 0.1% saponin/3% goat serum/PBS. Nuclei were stained for 20 min at rt with 10 μg/ml Hoechst 33342 (Sigma-Aldrich) in PBS. Unbound fluorescent conjugate was removed by three washes with 0.1% saponin/PBS, cells mounted in Vectashield (Vector Laboratories) and viewed with a fluorescent microscope (Nikon, Eclipse TE300).

EXAMPLE 8: Metabolic labeling of proteins and immunoprecipitation.

Cell monolayers in 35-mm diameter wells were incubated for 0.5-10 h in methionine- and -cysteine-deficient MEM containing 1/40th the normal concentration of methionine, 5% dialyzed FBS and Express ^{35}S-protein labeling mix (140 μCi/ml; NEN). Labeled cells were washed once with cold PBS and harvested in 200 μl of sodium dodecyl sulfate (SDS) lysis buffer (0.1 M sodium phosphate buffer (pH 7.0), 1% SDS, 1x complete protease inhibitor cocktail (Roche Applied Science), 80 μg phenylmethylsulfonyl fluoride (PMSF) per ml) and cellular DNA sheared by repeated passage through a 27.5-gauge needle. Equal amounts of protein lysates (50 μl) were heated at 75°C for 10 min and clarified by centrifugation prior to mixing with 200 μl of TNA (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.67% bovine serum albumin, 1 mM EDTA, 0.33% triton X-100, 80 μg of PMSF per ml). One-μl of HCV positive patient serum (JHF) was added, and immune complexes allowed to form by incubation overnight at 4°C with rocking. Immune complexes were collected by adding 50 μl of prewashed Pansorbin cells (Calbiochem) and incubation for 1-2 h at 4°C with rocking. Immunoprecipitates were collected by centrifugation and washed three times in TNAS (TNA containing 0.125% SDS) and once with TNE (50 mM

Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 80 μg of PMSF per ml), solubilized by heating at 80 °C for 20 min in protein sample buffer and separated on an SDS-10% polyacrylamide gel. Metabolically labeled proteins were visualized by fluorography.

EXAMPLE 9: Cell lines permissive for HCV replication.

From 22 G418-resistant clones (Blight, K.J. 2000), clones Huh-7.5 and Huh-7.8, harboring SG-Neo subgenomic replicons with no amino acid changes within the HCV NS region, as well as clone Huh-7.4, containing a replicon with the Ser to Ile change at position 2204 in NS5A, were cured. Uncloned population lines Huh-7/S2204I and Huh-7/5AΔ47 (Blight, K.J. 2000), selected with G418 after transfection of subgenomic replicons containing either S2204I in NS5A {SG-Neo (S2204I); Fig. 1} or the 47 amino acid NS5A deletion {SG-Neo (5AΔ47); Fig. 1}, were also treated with IFN. To exclude the possibility that IFN treatment alone may alter the ability of Huh-7 cells to support HCV replication, the parental Huh-7 cells were treated with IFN in parallel. Following IFN treatment, cells were shown to lack HCV RNA by a nested RT-PCR specific for the 3' NTR where the detection limit was ~10 molecules of HCV RNA (Kolykhalov, A.A., J. Virol. 70:3363-3371) and by sensitivity to G418.

To examine the ability of IFN-cured cell lines to support HCV replication, three G418-selectable replicons, SG-Neo (S2204I), SG-Neo (5AΔ47) and SG-Neo (wt) (Fig. 1), with G418-transduction efficiencies in parental Huh-7 cells of 10%, 0.2% and 0.0005%, respectively, were used (Blight, K.J. 2000). In vitro-synthesized RNA was electroporated into IFN-cured cells and after 48 h, G418 selection was imposed and the resulting colonies counted after fixing and staining. The transduction efficiencies were calculated on the basis of the number of G418-selected colonies relative to the number of Huh-7 cells plated after electroporation. The frequency of Huh-7.5 cells able to support SG-Neo (S2204I) replication was ~3-fold higher than the parental Huh-7 cells (Fig. 2). For cell lines Huh-7.5 and Huh-7.8, the number of G418-resistant colonies obtained after transfection of SG-Neo (5AΔ47) was significantly higher than for the parental Huh-7 cells (~33- and 9-fold increases, respectively; Fig. 2). The same was true for Huh-7.4, although the increased frequency of colony formation was not as great (~3-fold; Fig. 2). The SG-Neo (wt)

replicon also showed an enhanced replicative capacity in Huh-7.5, Huh-7.8 and Huh-7.4 cells (10-, 2- and 2-fold increases, respectively; Fig. 2).

The two cured cell populations, Huh-7/5AΔ47 and Huh-7/S2204I, showed either comparable or modest increases in G418 transduction efficiencies after transfection of the adapted replicon RNA originally present within the population line (Fig. 2). The frequency of G418-resistant colonies increased ~2.5-fold when Huh-7/5AΔ47 cells were electroporated with SG-Neo (5AΔ47), whereas transfection with SG-Neo (S2204I) resulted in a slight decrease in the G418 transduction efficiency (Fig. 2). However, a 23-fold reduction in colony formation was observed after transfection of Huh-7/S2204I cells with SG-Neo (5AΔ47) (Fig. 2). No significant differences in G418-resistant colony formation were noted between the parental Huh-7 cells and IFN-treated Huh-7 cells (data not shown), indicating that the IFN-mediated curing protocol did not stably influence the ability of these cells to support HCV replication. G418-resistant colonies were not observed when the polymerase defective replicon RNA, pol$^-$, was transfected in parallel (data not shown). Hence, a higher frequency of cells in the cured clonal lines, in particular those originally able to support replication of RNAs without adaptive mutations (Huh-7.5 and Huh-7.8), are permissive for HCV replication.

EXAMPLE 10: HCV Replication in unselected Huh-7.5 and Huh-7 cells.

Since the cured Huh-7.5 line was the most permissive of those tested, we examined HCV replication in this subline compared with the parental Huh-7 cells using a number of different methods. We focused on transient assays that would allow an assessment of HCV replication early after transfection without the need for G418 selection. Ninety-six hours after transfection with SG-Neo (S2204I) and SG-Neo (5AΔ47) RNA (Fig. 1), total RNA was extracted from Huh-7.5 and IFN-treated Huh-7 cells and the HCV RNA levels quantified by RT-PCR. The replication-defective replicon, pol$^-$, was transfected in parallel to allow discrimination between input RNA and RNA generated by productive replication. As shown in Fig. 3, the levels of HCV RNA relative to the pol$^-$ control were consistently higher in the transfected Huh-7.5 cells. Transfection with SG-Neo (S2204I) and SG-Neo (5AΔ47) RNAs resulted in 410- and 28-fold increases, respectively, in Huh-7.5 cells (Fig. 3,

lanes 4 and 5), compared to only 85- and 6-fold in Huh-7 cells (Fig. 3, lanes 10 and 11). Since these increases are measured relative to the replication defective pol- control, they reflect accumulation of newly synthesized RNA versus degradation of input RNA. In Huh-7.5 and Huh-7 cells, the level of residual pol- RNA declined by about 10-fold at each timepoint. In Huh-7.5 cells, RNAs was good replicative abilities {like SG-Neo (S2204I)} tended to accumulate over time such that about a 10-fold increase was observed by 96 h. Those with lower replicative ability {like SG-Neo (5AΔ47)} remained constant or declined slightly, but never to the level of the pol- control. For Huh-7 cells, the picture was somewhat different. For example, SG-Neo (S2204I) RNA remained relatively constant whereas SG-Neo (5AΔ47) RNA decreased over time, but again, not to the extent of the pol- control.

This finding was mirrored by the frequency of NS3-positive cells measured by FACS analysis. The percentage of NS3-positive cells was consistently higher in Huh-7.5 cells {21% for SG-Neo (S2204I) and 5% for SG-Neo (5AΔ47); Fig. 3, lanes 4 and 5} compared to Huh-7 cells {3% for SG-Neo (S2204I) and undetectable for SG-Neo (5AΔ47) and pol$^-$ RNAs; Fig. 3, lanes 7, 10 and 11}. These results confirm our earlier conclusion that a larger fraction of Huh-7.5 cells support detectable levels of HCV replication. The lower frequency of HCV antigen-positive cells quantified by FACS compared to the G418 transduction efficiency is attributable to the sensitivity of FACS analysis, that varies with different HCV-specific antibodies (unpublished observations).

HCV protein accumulation was examined by metabolically labeling cells 96 h after transfection. Cell monolayers were labeled with ^{35}S-methionine and -cysteine for 10 h, followed by SDS-mediated lysis and immunoprecipitation of HCV proteins with a HCV-positive patient serum (JHF) recognizing NS3, NS4B and NS5A (Grakoui, A. et al., J. Virol. 67:1385-1395). After separation of labeled proteins by SDS-PAGE, NS3, NS4B and NS5A were only visible in Huh-7.5 cells transfected with SG-Neo (S2204I) (Fig. 3, lane 4). HCV proteins were never detected in Huh-7.5 and Huh-7 cells transfected with SG-Neo (5AΔ47), pol$^-$ or SG-Neo (S2204I) RNA electroporated Huh-7 cells (Fig. 3, lanes 1, 5, 7, 10 and 11). Similar results were obtained after metabolic labeling of HCV RNA in the presence of actinomycin D (data not shown). These analyses demonstrate the advantages of using Huh-7.5 cells

for rapid analysis of HCV replication by RNA accumulation, FACS analysis and metabolic labeling of viral proteins.

EXAMPLE 11: Replicative efficiencies of subgenomic and genomic HCV RNAs.

The ability to monitor HCV replication without selection eliminated the need for bicistronic replicons and allowed constructs with minimal heterologous elements to be tested. A subgenomic replicon was engineered in which the HCV 5' NTR and 12 amino acids of core were fused to ubiquitin followed by the NS3-5B coding region (including the S2204I adaptive mutation in NS5A) and the 3' NTR {SG-5'Ub-NS3 (S2204I); Fig. 1}. In this polyprotein, cellular ubiquitin carboxyl-terminal hydrolase will cleave at the ubiquitin/NS3 junction to produce NS3 with an authentic N-terminal Ala residue (2, 21). In vitro-synthesized RNA was electroporated into Huh-7.5 and Huh-7 cells and the level of HCV RNA quantified 96 h later by RT-PCR. Surprisingly, HCV RNA levels did not differ from the pol$^-$ control (data not shown), indicating that SG-5'Ub-NS3 (S2204I) RNA failed to replicate. It is possible that ubiquitin may interfere with the production of a functional NS3 protein. However, a bicistronic derivative, where expression of ubiquitin/NS3-5B was under the control of the EMCV IRES, replicated as efficiently as SG-Neo (S2204I) RNA (data not shown), suggesting that HCV IRES driven translation may be sensitive to RNA elements present within the core-ubiquitin coding sequence.

A replicon lacking *neo* but retaining the EMCV IRES (SG-5'HE derivatives; Fig. 1) was also tested. SG-5'HE (S2204I) and SG-5'HE (5AΔ47) RNA levels relative to the pol$^-$ control were measured 96 h after transfection and found to be higher than the selectable versions in both Huh-7.5 and Huh-7 cells (Fig. 3). Moreover, the levels of SG-5'HE (S2204I) and SG-5'HE (5AΔ47) RNA were significantly higher in Huh-7.5 compared to Huh-7 cells (Fig. 3, lanes 2, 3, 8 and 9). Approximately 52% of Huh-7.5 cells stained positive for the NS3 antigen after transfection of SG-5'HE (S2204I) RNA, compared to 21% for SG-Neo (S2204I) RNA (Fig. 3, lanes 2 and 4). Similarly, a higher frequency of Huh-7.5 cells expressed NS3 after electroporation with SG-5'HE (5AΔ47) compared to SG-Neo (5AΔ47) (11% versus 5%; Fig. 3, lanes 3 and 5). As expected, lower frequencies of NS3-positive cells were observed for transfected Huh-7 cells (Fig. 3). The relative amounts of immunoprecipitated ^{35}S-labeled HCV

proteins from Huh-7.5 and Huh-7 cells paralleled both the frequency of NS3-positive cells and relative HCV RNA levels (Fig. 3). These data demonstrate that replicons lacking the *neo* gene initiate RNA replication more efficiently. These constructs, together with the highly permissive Huh-7.5 subline, are valuable tools for genetic studies on HCV RNA replication, some of which are described later in this report.

The ability of Huh-7.5 cells to support replication of full-length HCV RNA containing S2204I in NS5A {FL (S2204I); Fig. 1} was also assessed. Ninety-six hours after transfection of Huh-7.5 and Huh-7 cells, the relative levels of HCV RNA and protein were measured as described above. A 50-fold increase in HCV RNA relative to pol⁻ was observed after transfection of Huh-7.5 cells, compared to only a 3-fold increase in Huh-7 cells (Fig. 3, lanes 6 and 12). Similarly, FACS analysis and immunoprecipitation of metabolically labeled proteins failed to detect HCV antigen expression in FL RNA transfected Huh-7 cells, whereas 14% and 10% of Huh-7.5 cells expressed core and NS3 antigens, respectively, and ^{35}S-labeled NS3 was detectable (Fig. 3, lanes 6 and 12). The frequency of core-antigen positive cells was consistently higher than that seen for NS3, possibly reflecting differences in antibody affinity. The ability of full-length HCV RNA to establish replication in Huh-7.5 cells demonstrates that replication is not dependent upon EMCV IRES-driven translation of HCV-encoded replicase components. In fact, inclusion of the EMCV IRES downstream of the HCV 5' NTR {FL-5'HE (S2204I); Fig. 1} or creation of a biscistronic construct with the *neo* gene added {FL-Neo (S2204I); Fig. 1} impaired replication relative to FL (2204I)RNA (Fig. 4). It is interesting to note that all of the constructs containing the complete HCV coding sequence (S2204I-containing FL, FL-5'HE and FL-Neo) were less efficient at establishing replication compared to subgenomic replicons lacking the structural-NS2 coding region {eg. SG-5'HE (S2204I); Fig. 4}. This suggests that *cis* RNA elements or proteins encoded in this region of the genome may downregulate the efficiency of HCV replication in this system. Nonetheless, the ability of Huh-7.5 cells to support replication of both FL and FL-Neo RNAs provides systems that may be useful for studying steps in particle assembly and examining the impact of the entire HCV protein complement on host cell biology.

EXAMPLE 12: Effect(s) of mutations in NS3 and NS5A on HCV RNA replication,

Thus far, the best single mutation that has been identified is the S2204I substitution in NS5A . To examine the importance of Ile at this position and to see if replication efficiency could be improved further, a number of other amino acids were tested at this position and compared the replication efficiency of these replicons to SG-5'HE (S2204I) or the unmodified parent, SG-5'HE (S2204)(Fig. 5). Replicative ability was assessed in RNA transfected Huh-7.5 cells by comparing the HCV RNA levels to a SG-pol$^-$ control. Comparable levels of HCV RNA were observed at 96 h for replicons containing Ile or Val at position 2204, whereas an Ala substitution resulted in a 3-fold reduction in HCV RNA compared to SG-5'HE (S2204I) (Fig. 5). In contrast, the remaining amino acid substitutions dramatically reduced HCV RNA to levels similar to the unmodified parental replicon, SG-5'HE (S2204) (~1400-fold decrease; Fig. 5). As expected, the relative HCV RNA levels were lower at 24 and 48 h after transfection, however the levels were sufficient to assess replicative ability at 48 h (Fig. 5). Although substitutions that enhance subgenomic replication above that observed with S2204I were not found, Val and Ala allowed efficient RNA replication.

The replication efficiency of subgenomic replicons carrying multiple adaptive mutations in NS5A was investigated. NS5A mutations S2197P, A2199T and S2204I independently enhance G418-resistant colony formation approximately 2,500-, 15,000- and 20,000-fold, respectively (5). SG-5'HE replicons (Fig. 1) carrying S2204I together with either A2199T, or A2199T and S2197P were constructed and HCV RNA levels in Huh-7.5 cells measured by RT-PCR. Combining these NS5A mutations led to a reduction in HCV RNA levels compared to SG-5'HE (S2204I), with a 13-fold decrease for the combination of A2199T and S2204I and negligible replication when all three were combined (Fig. 6). Despite the observation that each of these NS5A adaptive mutations alone enhanced replication, when combined, the replicative ability of subgenomic RNAs declined, suggesting that these combinations are incompatible.

NS3 changes at positions 1112 (Q to R), 1202 (E to G) and 1280 (T to I) were engineered into SG-5'HE (S2204I) (Fig. 1) and their replication compared in Huh-7.5 cells by measuring HCV RNA levels, the frequency of antigen-positive cells and by detection of ^{35}S-labeled proteins at 96 h following transfection. Equivalent levels of

HCV RNA relative to the pol⁻ RNA control were observed for each replicon (Fig. 7A). The percentage of NS5B-positive cells detected by FACS (~30%; Fig. 7A) and immunofluorescence (Fig. 7B) was also similar. However, the frequency of NS3-positive cells was higher for replicons carrying the NS3 mutations (~73-87%; Fig. 7A), which may simply reflect altered affinity of the NS3-specific antibody for these NS3 mutants. Finally, the levels of immunoprecipitated NS3, NS4B and NS5A were comparable (Fig. 7A). Although it was not verified that Q1112R alone was adaptive, Krieger and coworkers previously reported that E1202G and T1280I alone or together increased the replication efficiency by ~13-, 6- and 25-fold, respectively (Krieger et al. 2001). These NS3 adaptive mutations do not further enhance replication when combined with S2204I in NS5A.

EXAMPLE 13: Mutagenesis of the S2194 NS5A phosphorylation site.

The role of NS5A phosphorylation in HCV replication remains a mystery. Previously, differences were noted in the extent of NS5A phosphorylation between replicons with different adaptive mutations in NS5A (Blight et al. 2000). For example, replicons with S2197C, S2197P or S2204I expressed minimal or no p58 as assessed by one-dimensional SDS-PAGE separation of immunoprecipated NS5A, suggesting that NS5A hyperphosphorylation is not essential for HCV replication. Recently, S2194 in NS5A of a subtype 1b isolate was identified as the primary site of p56 phosphorylation (Katze et al. 2000). To assess the possible requirement for phosphorylation of NS5A S2194, this residue was mutated in SG-Neo (S2204I) (Fig. 1) to either Ala (S2194A+S2204I) or Asp (S2194D+S2204I), to ablate or mimic phosphorylation, respectively. G418 transduction efficiencies of these replicons in Huh-7 cells was significantly lower than SG-Neo (S2204I) (120- and 17-fold lower; Fig. 8A). To rule out the possibility that G418-resistant foci were generated by reversion at this locus, the NS5A coding region was amplified from total cellular RNA by RT-PCR and directly sequenced. The original Ala and Asp substitutions at position 2194 were confirmed (data not shown). To minimize the impact of possible second site compensating changes, HCV RNA and protein expression was measured 96 h after RNA transfection of Huh-7.5 cells. The HCV RNA levels of S2194A+S2204I and S2194D+S2204I relative to the pol⁻ control were ~37- and 5-

fold lower than SG-Neo (S2204I) (Fig. 8B), consistent with their reduced ability to render Huh-7 cells G418 resistant. In addition, a lower frequency of NS5B-positive cells was evident in S2194A+S2204I than in S2194D+S2204I (data not shown), and ^{35}S-labeled NS3 and NS4B were only detectable in Huh-7.5 cells transfected with SG-Neo (S2204I) and S2194D+S2204I (Fig. 8B). It was not possible to directly study the phosphorylation status of NS5A expressed from S2194A+S2204I and S2194D+S2204I since the levels of NS5A expressed in transiently transfected cells were below the detection limit (Fig. 8B and data not shown). Although the quantitative differences in G418 transduction and replication efficiencies are difficult to interpret given the possible incompatibility of combining the S2194 substitutions with the S2204I adaptive change, these data show that phosphorylation of S2194 is not an absolute requirement for HCV replication.

Figure Legends

FIG. 1. Schematic representation of HCV RNAs used in this study. The 5' and 3' NTR structures are shown and ORFs depicted as open boxes with the polyprotein cleavage products indicated. The first 12 amino acids of the core-coding region (solid box), the *neo* gene (Neo; shaded box), the EMCV IRES (EMCV; solid line) and ubiquitin (hatched box) are illustrated. Locations of the NS5A adaptive mutations S2204I (*) and Δ47aa are indicated.

FIG. 2. Identification of Huh-7 lines highly permissive for HCV replication. Huh-7 cells that had been cured of self-replicating subgenomic RNAs by extended IFN treatment were electroporated with 1 μg of the subgenomic replicons, SG-Neo (S2204I), SG-Neo (5AΔ47) and SG-Neo (wt). Forty-eight hours later, cells were subjected to G418 selection and the resulting colonies fixed and stained with crystal violet. Representative plates are illustrated with the number of transfected cells seeded per 100-mm diameter dish shown on the left. Numbers below each dish refer to the calculated G418 transduction efficiency of the replicon. To determine the G418 transduction efficiency, transfected cells were serially titrated from 5x 10^5 to 10^3 cells per 100-mm diameter dish, together with feeder cells electroporated with the pol$^-$ replicon. The resulting G418-resistant foci were counted for at least 3 cell densities and the relative G418 transduction efficiency expressed as a percentage, after dividing

the number of colonies by the number of electroporated cells initially plated. Similar transduction efficiencies were obtained in two independent transfections. A poliovirus subgenomic replicon expressing GFP (see Methods) was electroporated in parallel. Based on both the fraction of GFP-positive cells and replicon-induced cytopathogenicity, ~90% of cells were routinely transfected. NT = not tested

FIG. 3. Detection of HCV proteins and RNA in Huh-7.5 and Huh-7 cells transiently transfected with HCV RNA. Top panel, Huh-7.5 and Huh-7 cells were transfected with the subgenomic replicons, pol$^-$ (lanes 1 and 7), SG-5'HE (S2204I) (lanes 2 and 8), SG-5'HE (5AΔ47) (lanes 3 and 9), SG-Neo (S2204I) (lanes 4 and 10), SG-Neo (5AΔ47) (lanes 5 and 11) and FL (S2204I) HCV RNA (lanes 6 and 12). At 96 h posttransfection, monolayers were incubated for 10 h in the presence of ^{35}S-methionine and -cysteine. Labeled cells were lysed, immunopreciptated with HCV-positive human serum (JHF, anti-NS3, NS4B and NS5A) and labeled proteins separated by SDS-10% PAGE. Note that twice the amount of immunopreciptated sample was loaded in lanes 6 and 12 (2x). The mobilities of molecular mass standards (MW) are indicated on the left and the migration of NS3, NS4B, NS5A and 5AΔ47 are shown on the right. Middle panel, Total cellular RNA was extracted at 96 h posttransfection and quantified for HCV RNA levels as described in the Materials and Methods. The ratio of HCV RNA relative to the pol$^-$ defective replicon is shown (HCV RNA/pol$^-$). HCV RNA levels relative to the pol$^-$ control were comparable in three independent experiments. Lower two panels, 96 h after transfection cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, stained for either HCV core or NS3 antigens and analyzed by FACS. The percentage of cells expressing core and NS3 relative to an isotype matched irrelevant IgG is displayed. Values <1.5% were considered negative (-).

FIG. 4. HCV RNA accumulation after transfection of Huh-7.5 cells with full-length HCV RNA. One-μg of in vitro transcribed RNA was electroporated into Huh-7.5 and 2 x 10^5 cells plated into 35-mm diameter wells. Total cellular RNA was isolated at 24, 48 and 96 h posttransfection and HCV RNA levels quantified as described in the Materials and Methods. The ratio of HCV RNA relative to the pol$^-$ defective subgenomic RNA (HCV RNA/pol$^-$) was plotted against the time posttransfection and similar results were obtained when this experiment was repeated

FIG. 5. Effects of alternative substitutions at position 2204 on HCV RNA replication. Huh-7.5 cells were transfected with 1 μg of the SG-5'HE replicons carrying the indicated amino acid substitutions and 2 x 10^5 cells plated in 35-mm diameter wells. After 24, 48 and 96 h in culture, total cellular RNA was extracted and HCV RNA levels measured as described in the Materials and Methods. The ratio of HCV RNA relative to the pol⁻ defective subgenomic RNA (HCV RNA/pol⁻) was plotted against the time posttransfection. The increase in HCV RNA above pol⁻ is indicated above each bar. In this figure the levels of HCV RNA relative to the pol⁻ are the highest we have achieved so far. When these RNAs were transfected into Huh-7.5 cells a second time a similar trend in HCV RNA accumulation was observed.

FIG. 6. Effect(s) of combining NS5A adaptive mutations on HCV RNA replication. Subgenomic replicons were transfected into Huh-7.5 cells and HCV RNA levels quantitated as described in Fig. 5. The ratio of HCV RNA relative to the pol⁻ defective subgenomic RNA (HCV RNA/pol⁻) was plotted against the time posttransfection and the increase in HCV RNA above pol⁻ is indicated above each bar. An additional transfection experiment yielded HCV RNA/pol⁻ ratios similar to those illustrated here.

FIG. 7. Effect(s) of combining NS3 and NS5A mutations on HCV RNA replication. Subgenomic replicons lacking *neo* were generated carrying S2204I with further mutations in NS3. (A) Top, 96 h after RNA transfection of Huh-7.5 cells, monolayers were labeled with ^{35}S-protein labeling mixture, lysed and NS3, NS4A and NS5A analyzed by immunoprecipitation, SDS-10% PAGE and autoradiography. Positions of the molecular weight standards are given on the left and HCV-specific proteins indicated to the right. Middle, Total cellular RNA was extracted at 96 h posttransfection and HCV RNA levels quantified as described in the Materials and Methods. The ratio of HCV RNA relative to the pol⁻ negative control is shown (HCV RNA/pol⁻). Comparable ratios were obtained in two independent experiments. Lower two panels, 96 h after transfection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, stained for HCV NS3 and NS5B antigens and analyzed by FACS. The percentage of cells expressing NS3 and NS5B relative to an isotype matched irrelevant IgG is displayed. Values <1.5% were considered negative (-). (B) Transfected cells seeded in eight-well chamber slides

were fixed, permeablized and stained for NS5B by immunofluorescence after 96 h in culture. Nuclei were counterstained with Hoescht 33342 and stained cells visualized by fluorescent microscopy (x40 magnification).

FIG. 8. Effect of S2194A and S2194D mutations on HCV RNA replication. S2194 was replaced with Ala or Asp in the selectable bicistronic replicon SG-Neo (S2204I) and RNA transcribed in vitro. (A) RNA transcripts were transfected into Huh-7 cells and G418-selected colonies fixed and stained with crystal violet. The relative G418 transduction efficiencies are indicated below each dish. (B) Ninety-six hours posttransfection Huh-7.5 cells were labeled with ^{35}S-methionine and -cysteine for 10 h. Cells were lysed, and HCV proteins isolated by immunoprecipitation using a patient serum specific for NS3, NS4B and NS5A. HCV proteins and the positions of protein molecular weight standards (in kilodaltons) are shown. The ratio of HCV RNA relative to the pol$^-$ negative control at 96 h posttransfection is shown below each track (HCV RNA/pol$^-$). The results illustrated are representative of two independent transfections.

As various modifications could be made in the constructions and methods herein described and illustrated without departing from the scope of the invention, it is intended that all matter contained in the foregoing description or shown in the accompanying drawings shall be interpreted as illustrative rather than limiting. Thus, the breadth and scope of the present invention should not be limited by any of the above-described exemplary embodiments.

TABLE 1. Oligodeoxynucleotides used in this study.

Name	Sequence
885	(-)CCCTCTAGAACGCCCCGAAACCTAGGGTGGCG
1030	(-)CCCTCTAGACTCGAGGGAATTTCCTGGAC
1184	(+)GACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTGACCAGCTGTCTGCGCCTTCC
1287	(+)AGACCGTGCACCAGACCACAACGGTTTCCCTCTAGCGGGATCAATTCCG
1288	(-)CCAGTAACGTTAGGGGGGGGGGAGGGAGAGGGGCGGAATTGATCCCGCT
1289	(+)CCAAAGGGCGCGCCATGCAGATCTTCGTGAAGACC 3'
1290	(-)AATAGGAGCTCCACCGCGGAGACGC
1291	(+)CGGTGGAGCTCCTATTACGGCCTACTCCCAAC
1292	(-)ATTGGTGTACATTTGGGTGATTGG
1293	(+)TCTGGAAGCTTCTTGAAGACA
1294	(-)GGCTTGACGTCCTGTGGGCGGCGGTTGGTGTTACGTTTGGTTTTTCTTTGAGGTTTAGGATTCGTGCTCATTATTATCGTGTTTTTCAAAGG
1319	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTGTACAGCTGTCTGCGCCTTCC
1320	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTGCCCAGCTGTCTGCGCCTTCC
1322	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTTACCAGCTGTCTGCGCCTTCC
1324	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTGAACAGCTGTCTGCGCCTTCC
1325	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGGCCAGCTCATCAGCTACACAGCTGTCTGCGCCTTCC
1326	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCTCCTTGACCAGCTCATCAGCTATCCAGCTGTCTGCGCCTTCC
1327	(+)AGACGGCTAAGCGTAGGCTGGCCAGGGGATCTCCCCCCCCCTTGACCAGCTCATCAGCTATCCAGCTGTCTGCGCCTTCC
1356	(-)CCGCTCTAGATACGTGATGGGGGCACCCGTGGTGATGGTCCTTACCCCGATTCTGATGTTAGGGTCGATAC
1358	(+)CCGATGTACACCAATGTGGACCAGGACCTCGTCGGCTGGCGAGCGCCCCCCGGGGCGCGTTCC
1359	(+)CCGCGTGCACCCGAGGGGTTGCGAAGGCGGTGGACTTTGTACCCGTCGAGTCTATGGGAACCACTATGCGGTCCCCGGTC
5'Ala	(+)CCACGCTAAGCGTAGGCTGGCCAGGGGAGCACCCCCCTCCTTGGCCAGCTC
5'Asp	(+)CCACGCTAAGCGTAGGCTGGCCAGGGGAGATCCCCCCTCCTTGGCCAGCTC

[a]Nucleotide changes are highlighted in bold and the resultant codon is underlined

[b]Restriction sites used for cDNA cloning are underlined

[c]The polarities of oligonucleotides are indicated either the HCV genome RNA sense (+) or its complement (-)